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Three Types of Taxis Used in the Response of *Acidovorax* sp. Strain JS42 to 2-Nitrotoluene

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***Acidovorax* sp. strain JS42 is able to utilize 2-nitrotoluene (2NT) as its sole carbon, nitrogen, and energy source. We report here that strain JS42 is chemotactic to 2NT and that the response is increased when cells are grown on compounds such as 2NT that are known to induce the first step of 2NT degradation. Assays with JS42 mutants unable to oxidize 2NT showed that the first step of 2NT metabolism was required for the induced response, but not for a portion of the constitutive response, indicating that 2NT itself is an attractant. The 2NT metabolite nitrite was shown to be a strong attractant for strain JS42, and sufficient nitrite was produced during the taxis assay to account for a large part of the induced response. A mutant with an inactivated *ntdY* gene, which is located adjacent to the 2NT degradation genes and codes for a putative methyl-accepting chemotaxis protein, showed a defect in taxis toward 2NT that may involve a reduced response to nitrite. Responses of a mutant defective for the energy-taxis receptor, Aer, indicated that a functional *aer* gene is required for a substantial part of the wild-type induced response to 2NT. In summary, strain JS42 utilizes three types of taxis to sense and respond to 2NT: constitutive 2NT-specific chemotaxis to directly sense 2NT, metabolism-dependent nitrite-specific chemotaxis that may be mediated by NtdY, and energy taxis mediated by Aer.**

Nitroarene compounds are a group of primarily man-made toxic chemicals known to contaminate the soil and groundwater of various locations around the world, including chemical manufacturing plants and munitions production and detonation sites (17, 68). Several strains of bacteria have been isolated that are able to mineralize selected nitroarene compounds (15, 16, 36–38, 44, 48, 55). The enzymes and pathways used to degrade these toxic chemicals have been the subject of several studies and are quite well understood (reviewed in references 24, 54, and 59).

Acidovorax (formerly *Pseudomonas* [32]) sp. strain JS42 was isolated from a nitrobenzene-contaminated site for its ability to utilize the toxic man-made nitroarene compound 2-nitrotoluene (2NT) as its sole carbon, nitrogen, and energy source (16). In strain JS42, 2NT degradation occurs by dioxygenation of the aromatic ring by 2-nitrotoluene 2,3-dioxygenase (2NTDO [4, 43]), resulting in formation of 3-methylcatechol (3MC) and nitrite. The aromatic ring of 3MC is then cleaved at the *meta* position by a catechol 2,3-dioxygenase, and the product is further degraded to compounds that enter the tricarboxylic acid (TCA) cycle (16) (Fig. 1). Expression of the *ntd* operon (*ntdAaAbAcAd*, encoding 2NTDO) is controlled by the transcriptional activator NtdR, which is encoded by a gene located directly upstream of and divergently transcribed from the *ntd* operon (32). NtdR activates transcription of the *ntd* operon in response to a broad set of aromatic inducer compounds, including 2NT and the gratuitous inducer salicylate, but not the 2NT metabolites 3MC and nitrite (22, 32).

Although the 2NT degradation pathway has been well studied, the ability of *Acidovorax* sp. JS42 to detect and respond to 2NT by the use of chemotaxis has not been investigated. Chemotaxis is a widespread behavior in which cells respond to environmental stimuli by altering the direction of net cellular movement. The stimulus profile to which a bacterium responds is dictated by the specificity of chemoreceptor proteins, which are often membrane-bound methyl-accepting chemotaxis proteins (MCPs; reviewed in references 19, 27, 35, and 63). Chemotaxis has been shown to increase the rate of degradation of specific chemical compounds that serve as carbon and energy sources, presumably by overcoming obstacles such as spatial separation of bacteria and pollutant (29, 34, 47, 58, 64). Thus, understanding details of the mechanism of compound detection, such as the role of metabolism and identification of receptors involved in the tactic response to a pollutant, could contribute to the more efficient removal of that pollutant from contaminated sites. The goal of this work was to characterize the chemotactic response of *Acidovorax* sp. strain JS42 toward 2NT, including identification of the specific attractants detected and the role of 2NT metabolism in the response. Results presented here demonstrate that strain JS42 is chemotactic to 2NT itself, as well as to the nitrite it generates during 2NT degradation. In addition, JS42 uses energy taxis to sense the energy obtained during growth on 2NT.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. Prior to initiation of chemotaxis experiments, the motility of *Acidovorax* sp. strain JS42 cells was optimized by transferring the cells several times in tryptone-yeast extract (TY) soft agar medium (0.1% tryptone, 0.05% yeast extract, 0.3% Bacto agar) as previously described (62). The resulting motile strain (JS42m) was used for all experiments, and all mutants generated in this study were derived from this motile strain. *Escherichia coli* DH5α/λpir (65) was used for cloning and plasmid propagation. *E. coli* S17-1 (53) was used as a donor strain in conjugation experiments; in some cases, HB101(pRK2013) (11) was used as a helper strain to mobilize plas-

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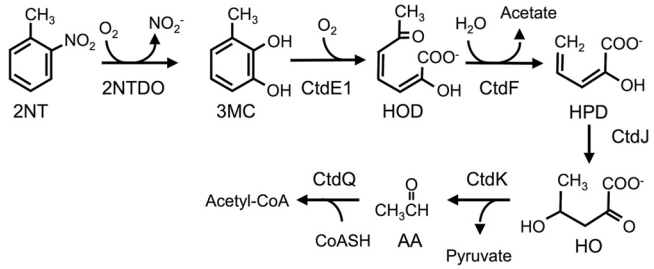

FIG 1 2-Nitrotoluene (2NT) degradation pathway in *Acidovorax* sp. strain JS42. 2NTDO, 2-nitrotoluene 2,3-dioxygenase, encoded by *ntdAaAbAcAd*. The catechol degradation (Ctd) enzymes were identified based on the analysis of the JS42 genome sequence (23). CtdE1, catechol 2,3-dioxygenase; CtdF, 2-hydroxymuconate semialdehyde hydrolase; CtdJ, 2-oxopent-4-dienoate hydratase; CtdK, 4-hydroxy-2-oxovalerate aldolase; CtdQ, acetaldehyde dehydrogenase (acylating); 3MC, 3-methylcatechol; HOD, 2-hydroxy-6-oxohepta-2,4-dienoate; HPD, 2-hydroxypenta-2,4-dienoate; HO, 4-hydroxy-2-oxovalerate; AA, acetaldehyde.

mids in triparental matings. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (50). Matings between *E. coli* and *Acidovorax* strains were carried out at 30°C on TY plates (1% tryptone, 0.5% yeast extract, 1.8% Bacto agar) for 48 to 72 h. *Acidovorax* strains were grown in minimal salts broth (MSB) (56) containing succinate (10 mM) and Balch's vitamin solution (12) lacking thiamine and *p*-aminobenzoic acid. Noble agar (Difco, Sparks, MD) (1.8%) was used to solidify MSB for plates. Vaporous 2NT was provided as a carbon source by taping a culture tube (6 by 50 mm) containing 25 μl of 2NT to the lid of MSB plates. For plasmid selection and maintenance, antibiotics were added at the following concentrations: ampicillin at 200 μg/ml, chloramphenicol at 30 μg/ml, gentamicin at 15 μg/ml, kanamycin at 50 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 20 μg/ml. To induce cells, cultures were grown to mid-exponential phase (when the optical density at 660 nm [OD_{660}] was approximately 0.25), and the inducer compound was added directly to the culture from a 1,000× stock solution, to reach a final concentration of 500 μM. 3-Methylcatechol (3MC), 2-nitrobenzyl alcohol (2NBA), and nitrite stock solutions were made in water, and 2NT and salicylate stock solutions were made in methanol.

DNA manipulations. Standard methods were used to manipulate plasmids and DNA fragments (50). *E. coli* strains were transformed with plasmid DNA according to standard methods (50). The primers used in

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Acidovorax sp.		
JS42	Wild type; $2NT^+$	16
JS42m	Wild type; $2NT^+$; selected for efficient motility	This study
CAR3	JS42m with insertionally inactivated *ntdY*; Km^r; $2NT^+$	This study
CAR5	JS42m with in-frame deletion of *aer*; $2NT^+$	This study
CAR12	JS42m with *ntdY*::Km and in-frame deletion of *aer*; Km^r; $2NT^+$	This study
CAR20	JS42m with insertionally inactivated *ntdAc*; Km^r; $2NT^-$; $3MC^+$	This study
CAR22	JS42m with insertionally inactivated *ctdE1*; Sm^r; $2NT^-$; $3MC^-$	This study
CAR24	JS42m with insertionally inactivated *cheA*; Km^r; $2NT^+$; motile; nontactic	This study
CAR26	JS42m with insertionally inactivated *ntdR*; Km^r; $2NT^-$; $3MC^-$	This study
E. coli		
S17-1	Plasmid mobilization strain	53
DH5α/λpir	Cloning host	65
Plasmids		
pAW19	*sacB*-containing cloning vector; Ap^r; Km^r	65
pBBR1MCS2	Broad-host-range vector; Km^r	26
pBBR1MCS5	Broad-host-range vector; Gm^r	26
pBBR1MCS5hc	pBBR1MCS5 with *rep* modified to increase copy no. per reference 61; Gm^r	This study
pCAR3	pAW19 containing *ntdY*::Km; Ap^r; Km^r	This study
pCAR4	pAW19 containing Δ*aer*; Ap^r; Km^r	This study
pCAR9	pBBR1MCS5 containing *aer*; Gm^r	This study
pCAR11	pBBR1MCS5hc containing *ntdAaAbAcAd* from pDTG800; Gm^r	This study
pCAR19	pRK415 containing most of *ntdY*; Tc^r	This study
pCAR20	pRK415 containing *ntdY*::Km; Tc^r	This study
pCAR27	pBBR1MCS5 containing *nbzY* from pDTG925; Gm^r	This study
pDTG800	pUC18 containing *ntdAaAbAcAd*; Ap^r	40
pDTG925	pUC18 containing 8.9-kb EcoRI fragment from *Comamonas* sp. strain JS765 that includes *nbzYRAaAbAcAd*	31
pDTG930	pRK415 containing *ctdE1*::Sm; Sm^r; Tc^r	22
pET28B	Cloning vector; Km^r	Novagen (Gibbstown, NJ)
pKSJ126	pBBR1MCS2 containing *ctdE1*; Km^r	22
pRK2013	ColE1 *ori*; RP4 mobilization function; Km^r	11
pRK415	Broad-host-range cloning vector; Tc^r	25
pRK415-850-Km	pRK415 containing *ntdAc*::Km; Km^r; Tc^r	22
pUC18	Cloning vector; Ap^r	67

[a] $2NT^+$, growth on 2NT; $2NT^-$, no growth on 2NT; $3MC^+$, growth on 3MC; $3MC^-$, no growth on 3MC; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

this study are shown in Table S1 in the supplemental material. *Pfu* high-fidelity polymerase with *Pfu* reaction buffer [200 mM Tris-Cl (pH 8.8), 100 mM $(NH_4)_2PO_4$, 100 mM KCl, 1% Triton X-100, bovine serum albumin (BSA) (1 mg/ml), 20 mM Mg_2SO_4] and standard conditions (95°C denaturing, 60°C annealing, and 72°C elongation temperatures and an elongation time of ~1 min per kilobase of PCR product) were used for all PCRs. Restriction and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Plasmids were purified using a commercial kit from Fermentas (Glen Burnie, MD), and genomic DNA was isolated using a commercial kit from Bioneer (Alameda, CA). DNA fragments were purified from gels by the use of a gel extraction kit from Fermentas. All PCR products were verified by fluorescent automated DNA sequencing at the University of California Davis Sequencing Facility with an Applied Biosystems 3730 automated sequencer.

Construction of strain CAR26. Strain CAR26, which lacks a functional *ntdR* gene, was constructed by triparental mating using DH5α(pDTG957) (32). The genotype was confirmed by PCR with relevant primers (32) (data not shown). For complementation, plasmid pNtd1 (32) carrying the *ntdR* gene was used.

Construction of strain CAR20. JS42m lacking a functional *ntdAc* gene was generated as previously described using DH5α(pRK415-850Km) (22). The genotype was verified by PCR using the nitroAc and nitroAd primers and the nitroAc and KanF EZTn5 primers (data not shown). For complementation, a high-copy-number variant of pBBR1MCS5 (pBBR1MCS5hc) was constructed as described by Tao et al. (61). The *ntd* operon (*ntdAaAbAcAd*; 4.9 kb) was excised from pDTG800 (40) by SacI digestion and ligated to pBBR1MCS5hc to generate pCAR11.

Construction of strain CAR22. JS42m lacking a functional *ctdE1* gene was constructed as previously described using DH5α(pDTG930) (22). The genotype was confirmed by Southern hybridization using the *ctdE1* gene as the probe (data not shown). For complementation, pKSJ126 (22) was introduced into CAR22 by conjugative mating from *E. coli* S17-1.

Construction of strain CAR3. A mutant with an insertionally inactivated *ntdY* gene (Ajs_3087) was constructed by PCR amplification of *ntdY* using primers 3087upF and 3087dnR and by cloning the 2.5-kb DNA fragment into the XbaI site of pRK415. The kanamycin resistance gene from pET28B was amplified by PCR using primers KanFMluI and KanRMluI, digested with MluI, and ligated to similarly digested pRK415*ntdY*. The fragment *ntdY*::Km was inserted into pAW19 by ligating XbaI-digested pRK415-*ntdY*::Km with SpeI-digested pAW19 to form pCAR3. Following a triparental mating with strains DH5α(pCAR3), HB101(pRK2013), and JS42m, kanamycin-resistant exconjugants were subjected to sucrose (20%) counterselection to identify variants that had lost the integrated pCAR3. Strain CAR3 was identified by PCR using primers 3087upF1 and 3087dnR6 and confirmed by Southern hybridization using the downstream DNA fragment as a probe (constructed with primers 3087dnF and 3087dnR6; data not shown). *nbzY*, which is 99% identical to *ntdY*, was cloned and used to complement strain CAR3. *nbzY* was excised from pDTG925 (31) by digestion with BamHI and SmaI, and the ~1.9-kb fragment was inserted into pBBR1MCS5 to generate pCAR27.

Construction of strain CAR5. Genomic DNA fragments of approximately 1 kb from upstream and downstream of *aer* (Ajs_3295) were PCR amplified using primers 3295upF and 3295upR and primers 3295dnF and 3295dnR, respectively. The PCR products were digested with BglII, ligated, and used as a template for PCR with primers 3295upF and 3295dnR. The 2.1-kb band was gel purified, digested with SacI and SpeI, and ligated with pAW19, forming pAW19Δ*aer*. Following a triparental mating with strains DH5α/λpir(pAW19Δ*aer*), HB101(pRK2013), and JS42m, kanamycin-resistant exconjugants were subjected to sucrose counterselection to obtain strain CAR5, which was verified by PCR using primers 3295upF and 3295dnR and confirmed by Southern hybridization (data not shown). For complementation, *aer* was PCR amplified using primers 3295upF Comp and 3295dnR Comp, digested with SacI and XbaI, and ligated with pBBR1MCS5, forming pCAR9.

Construction of strain CAR12. A double mutant lacking *aer* and carrying an insertionally inactivated *ntdY* gene was constructed by introduction of pCAR3 into strain CAR5 as described above for the generation of strain CAR3. The genotype of strain CAR12 was confirmed by PCR using primers 3087upF1 and 3087dnR6 and primers 3295upF1 and 3295dnR6 (data not shown).

Construction of strain CAR24. Primers cheAFor and cheAUp, cheADown and cheARev, and KanR and KanF were used to amplify approximately 900 bp of genomic DNA from upstream and downstream of *cheA* (Ajs_3790) and the kanamycin resistance gene from pET28B in order to generate a construct with an insertionally inactivated *cheA* gene. The construct was cloned into pRK415 and introduced into JS42m by conjugal mating from S17-1. A kanamycin-resistant, tetracycline-sensitive exconjugant was obtained, and interruption of *cheA* was confirmed by PCR with primers KanF and KanR, cheAFor and KanR, and cheARev and KanF (data not shown).

Qualitative capillary assays. Qualitative capillary assays (13) were used to monitor chemotaxis of strain JS42m and mutant derivatives. Cultures were harvested in the exponential phase (OD_{660} ~ 0.5 to 0.6) by centrifugation at 5,000 rpm for 3 min, washed once, and resuspended in chemotaxis buffer (50 mM potassium phosphate buffer [pH 7.0], 10 μM disodium EDTA, 0.05% glycerol) to an OD_{660} of approximately 0.2. Microcapillaries (Drummond Scientific Co., Broomall, PA) (1 μl) containing attractants in 2% low-melting-temperature agarose (NuSieve GTG; Lonza, Rockland, ME) dissolved in aerated chemotaxis buffer were introduced into suspensions of motile cells, and responses were visualized with a Nikon Eclipse TE2000-S microscope (Melville, NY) at ×40 magnification. Photographs were taken at 20-min intervals for 1 h using an Evolution Micropublisher 3.3 RTV camera (Media Cybernetics Inc., Silver Spring, MD) attached to the microscope and Evolution MP/QImaging software (Media Cybernetics Inc., Silver Spring, MD). Images shown here were recorded at 20 min unless otherwise noted. Prior to all assays, cell suspensions were evaluated microscopically to verify that ≥70% of cells were motile. All experiments were repeated at least three times and included positive (10 mM succinate) and negative (chemotaxis buffer) control capillaries. Consistently strong responses to the positive-control attractant confirmed that cells were fully motile and generally chemotactic and that differences in responses to test chemicals were due to either specific growth conditions or mutations.

Nitrite quantification assays. Nitrite generated from 2NT by cells during the qualitative capillary assay was quantified by determining the concentration of nitrite at the mouth of the capillary after 20 min, which was the typical peak response time. A 4-μl volume of cell suspension was removed from immediately in front of the mouth of the capillary by the use of a 20-μl pipette fitted with a thin protein gel loading pipette tip. Nitrite present in the sample was quantified using a scaled-down version of the nitrite assay (12) by monitoring absorbance with a Thermo Scientific NanoDrop 1000 spectrophotometer (Wilmington, DE) and comparison to a standard curve of $NaNO_2$. Each sample was assayed in triplicate in at least three independent experiments.

Soft agar swim plate assays. Soft agar plates were used to quantitatively measure taxis in MSB soft agar medium (18) containing 0.3% Noble agar, vitamins, and adipate (5 mM), succinate (10 mM), or 2NT (2 mM). *Acidovorax* strains were grown to the exponential phase (OD_{660} ~ 0.5 to 0.6) in MSB containing succinate and vitamins and washed and resuspended in chemotaxis buffer to an OD_{660} of approximately 0.4. Plates were inoculated by pipetting 2 μl of resuspended cells into the agar and incubated at 30°C. The diameters of the rings of swimming cells were measured using a "bucket of light" (45) approximately 24, 48, and 72 h after inoculation.

Growth studies. Growth rates of strain JS42m and its derivatives were determined in MSB containing vitamins with succinate (10 mM), 2NT (2 mM), or adipate (5 mM). Cultures were incubated at 30°C with shaking (200 rpm), and the optical density at 660 nm was monitored.

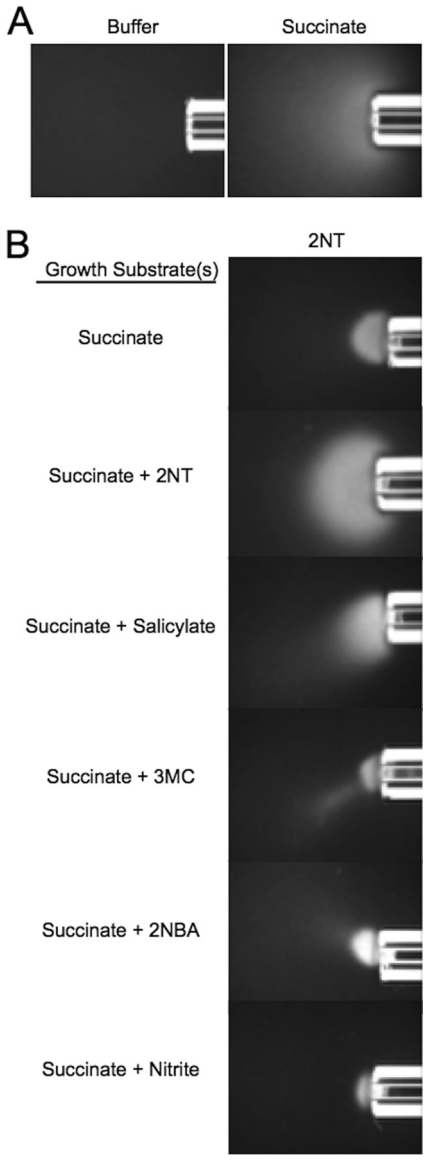

FIG 2 Wild-type JS42m responses in the qualitative capillary assay. Responses to buffer (negative control) or succinate (10 mM; positive control) (A) or to 2NT (saturated; ~4.4 mM) after growth with succinate alone (10 mM) compared to growth in the presence of possible inducers (B) are shown. The concentration of all inducer compounds was 500 μM. 3MC, 3-methylcatechol; 2NBA, 2-nitrobenzyl alcohol. The responses of all cultures to 10 mM succinate were comparable (data not shown).

RESULTS

***Acidovorax* sp. strain JS42 is constitutively chemotactic to 2NT, and the response is enhanced when cells are grown in the presence of 2NT.** Regardless of growth conditions, *Acidovorax* sp. strain JS42m showed no response to buffer alone (negative control) and a strong response to succinate (positive control) in qualitative capillary assays (Fig. 2A). When JS42m was grown with succinate (uninduced conditions), a consistent tactic response to 2NT was observed in qualitative capillary assays (Fig. 2B). When strain JS42m was grown in the presence of 2NT, a stronger response to 2NT was observed (Fig. 2B). Growth in the presence of salicylate, a strong inducer of the 2NT degradation genes (32), appeared to result in a weakly enhanced response to 2NT. In contrast, cells grown in the presence of 2-nitrobenzyl alcohol (2NBA; a dead-end metabolite of 2NT oxidation [41]), 3-methylcatechol (3MC), or nitrite did not show increased tactic responses to 2NT (Fig. 2B). 2NBA is a weak inducer and 3MC and nitrite are not inducers of the *ntd* operon (22, 32). Addition of these compounds to the medium did not affect the growth or motility of cells (data not shown). Despite significant efforts, attempts to quantify these relative responses using the quantitative capillary assay (1) were unsuccessful due to the strong tendency of JS42m to stick to glass and plastic surfaces, which resulted in highly variable data (data not shown).

JS42m is chemotactic to nitrite but not to other products of 2NT metabolism. In order to determine whether chemotaxis to intermediates of 2NT degradation could be contributing to the overall response to 2NT, we tested taxis to metabolites produced at various stages of 2NT degradation. We initially tested responses to 4.4 mM metabolites, as this is the limit of solubility of 2NT and therefore the maximum concentration of 2NT metabolites that could be produced during a 2NT capillary assay. Nitrite, a product of the first step in 2NT degradation, elicited a strong chemotactic response from JS42m, and the responses of uninduced and 2NT-induced JS42m toward nitrite were similar (Fig. 3A). In contrast, responses to even higher concentrations of 3MC and acetate (10 and 20 mM, respectively) were too weak to document, and no response to the dead-end metabolite 2NBA (4.4 mM) was detected (data not shown).

Since nitrite was found to be a strong chemoattractant, we wanted to determine whether sufficient nitrite to elicit a tactic response was generated from 2NT by induced and uninduced cells during a 20-min qualitative capillary assay. As expected, when 2NT (4.4 mM) was in the capillary and no cells were present, no nitrite was detected (data not shown). However, JS42m cells grown in uninduced conditions generated 4.0 $\pm$ 0.41 μM nitrite from 2NT, and 2NT-induced JS42m cells produced 9.7 $\pm$ 0.68 μM nitrite at the mouth of the capillary within 20 min. In order to determine whether these amounts of nitrite could elicit a response from JS42m, we first needed to establish how much nitrite within a capillary would allow accumulation of approximately 4 and 10 μM nitrite at the mouth of the capillary. By comparison to a standard curve of nitrite, we were able to determine that approximately 10 μM nitrite was present at the mouth of a capillary containing 2.5 mM nitrite after 20 min and that the presence of either induced or uninduced cells had no effect on the amount of nitrite that accumulated (Fig. 3B). This concentration of nitrite elicited a strong tactic response from JS42m, which is shown compared to the induced response to 2NT in Fig. 3C. Approximately 4 μM nitrite was present at the mouth of a capillary containing 0.5 mM nitrite (Fig. 3B), the response to which is shown compared to the uninduced response to 2NT (Fig. 3D). These comparisons suggest that nitrite formed from 2NT during a 20-min assay could account for a significant portion of both the induced and uninduced responses to 2NT.

Regulatory and metabolic mutants have defects in 2NT taxis and nitrite production compared to strain JS42m. In order to investigate the role of 2NT metabolism in taxis toward 2NT, we evaluated the responses of catabolic and regulatory mutants blocked at various steps in the degradation pathway. In addition, we determined the amounts of nitrite produced by each mutant

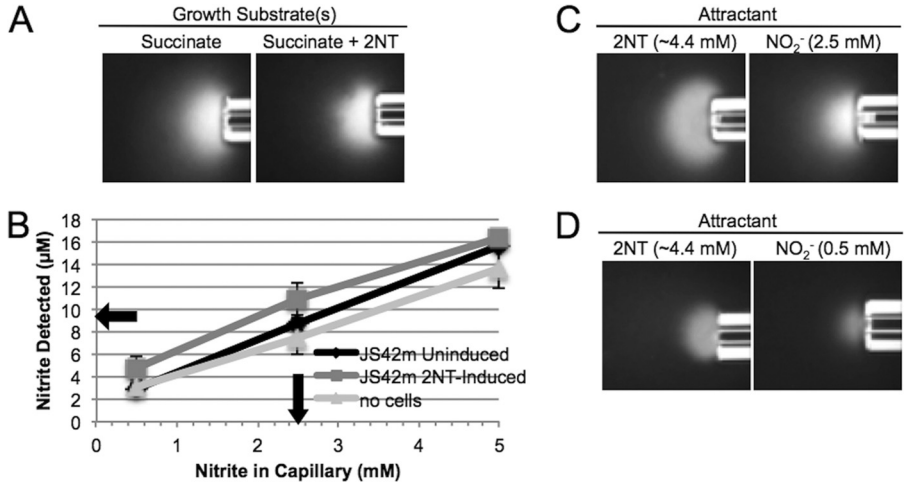

FIG 3 Chemotactic response to nitrite and generation of nitrite from 2NT during the qualitative capillary assay performed with wild-type JS42m. (A) Responses to ~4.4 mM nitrite by uninduced and 2NT-induced JS42m. (B) Determination of the amounts of nitrite accumulating at the tips of capillaries containing various nitrite concentrations after 20 min. Approximately 10 μM nitrite accumulated at the tip of a capillary containing 2.5 mM nitrite (arrows). The presence or absence of cells did not affect the accumulation of nitrite. The limit of detection of the nitrite assay is approximately 2 μM. (C) Comparison of induced responses of JS42m to 2NT (~4.4 mM) and 2.5 mM nitrite. The 2.5 mM nitrite in the capillary corresponds to the approximate amount of nitrite generated by induced cells from 4.4 mM 2NT during a 20-min assay. (D) Comparison of uninduced responses of JS42m to 2NT (~4.4 mM) and 0.5 mM nitrite. The 0.5 mM nitrite in the capillary corresponds to approximately 4 μM nitrite in solution at the mouth of the capillary (see panel B).

under the conditions of the chemotaxis assay in order to evaluate the contribution of nitrite to the response to 2NT.

Nitrite produced during the qualitative capillary assay was measured for the *ntdR*, *ntdAc*, and *ctdE1* mutants. Strain CAR26 lacks a functional NtdR, which activates transcription of the adjacent *ntd* operon encoding 2NTDO, the enzyme that catalyzes the first step of 2NT degradation (Fig. 1). Strain CAR20 has an insertionally inactivated *ntdAc* gene. As expected, the *ntdR* and *ntdAc* mutants generated no detectable nitrite during the qualitative capillary assay, regardless of growth conditions. Strains CAR26(pNtd1) (*ntdR* mutant expressing *ntdR* from the plasmid) and CAR20(pCAR11) (*ntdAc* mutant expressing *ntdAaAbAcAd* from the plasmid) generated nearly wild-type levels of nitrite in uninduced growth conditions but not during 2NT-induced growth (see Fig. S1 in the supplemental material). In qualitative capillary assays, *ntdR* mutant cells exhibited a very weak constitutive response to 2NT but were unable to mount an increased response to 2NT under induced conditions (Fig. 4). The complemented strain showed a restored wild-type phenotype, although its induced response took longer to peak than that of JS42m (see Fig. S1 in the supplemental material). In qualitative capillary assays, the *ntdAc* mutant remained capable of a very weak constitutive response to 2NT but was unable to mount the enhanced response after growth under induced conditions, which is similar to the results seen with the *ntdR* mutant (Fig. 4). The complemented *ntdAc* mutant showed a restored inducible response to 2NT, although the response was not of the intensity seen with wild-type JS42m (see Fig. S1 in the supplemental material). These results are consistent with the slightly lower growth rate of the complemented strain on 2NT compared to the wild type (data not shown).

Strain CAR22 lacks a functional *ctdE1* gene, which encodes the catechol 2,3-dioxygenase that catalyzes the second step of 2NT degradation (Fig. 1). The uninduced *ctdE1* mutant did not produce detectable amounts of nitrite from 2NT, but it generated

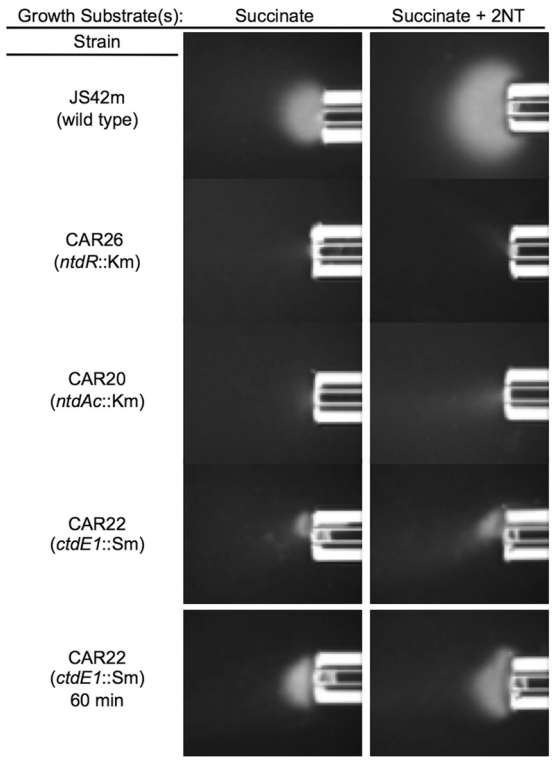

FIG 4 Chemotactic response to 2NT (~4.4 mM) in the qualitative capillary assay by JS42m and mutant derivatives following growth in uninduced and 2NT-induced conditions. Photographs were taken after 20 min. The response after 60 min is also shown for strain CAR22, since the peak response was delayed. All strains showed a strong response to succinate (positive control; data not shown).

5.7 ± 1.4 μM nitrite when grown in 2NT-induced conditions. The uninduced response of the *ctdE1* mutant to 2NT in the qualitative capillary assay was weaker than that of the wild type but stronger than that of the *ntdAc* mutant. Unlike the *ntdAc* and *ntdR* mutants, the response of the *ctdE1* mutant to 2NT was inducible but not to wild-type levels (Fig. 4). Although the peak response to 2NT for the uninduced *ctdE1* mutant occurred at 20 min, 2NT-induced cells displayed a peak response to 2NT at 60 min (Fig. 4). The delayed response could have been due to toxic effects of accumulating 3MC, but we did not test this possibility. The complemented *ctdE1* mutant showed a restored induced response but not a restored constitutive response (see Fig. S1 in the supplemental material). For reasons that are not clear, the complemented CAR22(pKSJ126) strain (*ctdE1* mutant expressing *ctdE1* from the plasmid), which is capable of growth on 2NT (data not shown), did not show a notable difference in nitrite production compared to the mutant strain (see Fig. S1 in the supplemental material). These partially complemented phenotypes are consistent with the partially restored growth phenotype on 2NT, as CAR22(pKSJ126) grew slower than the wild type on 2NT and accumulated brown-colored intermediates (data not shown).

Taken together, these data show that the strength of the chemotactic response to 2NT is generally correlated with the amount of nitrite produced in the wild type and in mutants defective in 2NT catabolism.

CheA is required for JS42m taxis toward 2NT. In chemotaxis, Che proteins transduce stimulus information to the flagellar motors (57). The genome sequence of *Acidovorax* sp. strain JS42 (GenBank accession no. NC 008782) contains homologs of *cheA*, *cheW*, *cheY*, *cheZ*, *cheB*, *cheR*, and *cheD* (see Table S2 in the supplemental material). CheA is a histidine kinase that initiates the chemotaxis phosphorelay controlling the direction of flagellar rotation in response to attractants and repellants. The genome of *Acidovorax* sp. strain JS42 has two *cheA* homologs (see Table S2 in the supplemental material). The product of Ajs_3790 is 62% identical to *E. coli* CheA and is located in an operon encoding a complete set of chemotaxis proteins; this gene was therefore chosen for inactivation. The mutant strain CAR24, which lacks a functional Ajs_3790 gene, was motile and displayed a smooth swimming phenotype, which is consistent with the behavior of *E. coli cheA* mutants (46). Regardless of growth conditions, this strain showed no tactic response toward 2NT (saturated; ~4.4 mM), nitrite (4.4 mM), or succinate (10 mM) in qualitative capillary assays (data not shown) (see Fig. S2 in the supplemental material); similarly, no response was seen in TY soft agar plates (see Fig. S2 in the supplemental material), indicating that the chemotactic responses of JS42m to these compounds depend on Ajs_3790 (*cheA*) function. Strain CAR24 was used as a generally nonchemotactic control strain in subsequent experiments.

NtdY is required for part of the JS42m response to 2NT. Analysis of the genome sequence of *Acidovorax* sp. strain JS42m revealed the presence of a gene, *ntdY* (Ajs_3087), encoding a putative MCP located adjacent to *ntdR*. The proximity of *ntdY* to the 2NT degradation genes made *ntdY* a good candidate to encode a receptor with a role in the detection of 2NT. The uninduced *ntdY* mutant (strain CAR3) showed a strong defect in the response to 2NT in qualitative capillary assays, while the 2NT-induced *ntdY* mutant cells showed an induced response that was less intense than that of 2NT-induced JS42m cells (Fig. 5). This mutant showed a response to nitrite that was similar to or slightly weaker

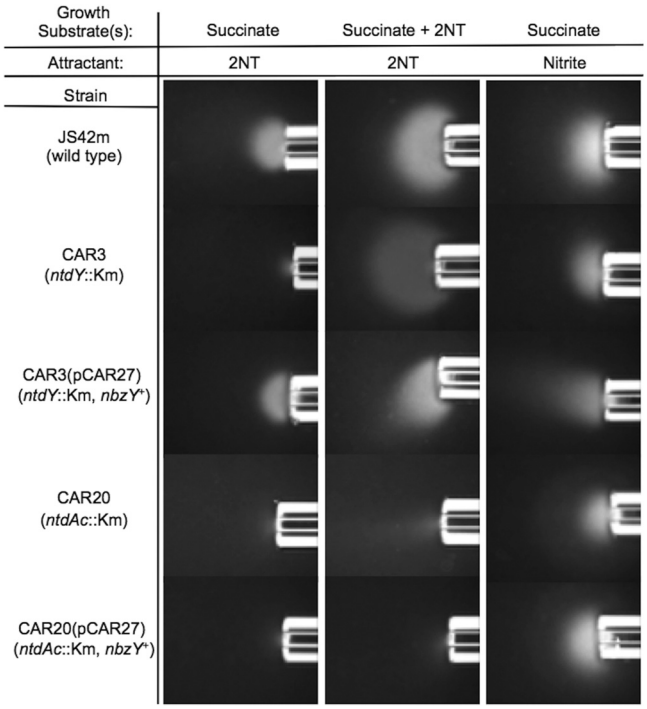

FIG 5 Tactic responses to 2NT (~4.4 mM) of the wild type (JS42m), *ntdY* mutant (CAR3), complemented *ntdY* mutant (CAR3[pCAR27]), *ntdAc* mutant (CAR20), and *ntdAc* mutant expressing the *nbzY* gene (CAR20[pCAR27]) in the qualitative capillary assay after growth in uninduced and 2NT-induced conditions and to the 2NT metabolite nitrite (4.4 mM) after growth in uninduced conditions. The responses of all strains to 10 mM succinate were comparable (data not shown).

than that of the wild type (Fig. 5). In contrast, the *ntdY* mutant response to succinate was consistently comparable to that of JS42m (data not shown).

High nucleotide sequence identity (99 to 100%) of approximately 5.8 kb of the DNA upstream of and including the first 42 bp of *ntdY* with four other regions of the genome of strain JS42 prevented successful PCR amplification of the complete *ntdY* gene. However, the nucleotide sequence of *ntdY* is 99% identical to *nbzY*, a putative MCP-encoding gene located upstream of the nitrobenzene dioxygenase-encoding *nbz* operon in *Comamonas* sp. strain JS765 (32). NbzY and NtdY differ by only one amino acid residue at position 56 (glutamine and lysine, respectively), which is located in the N-terminal ligand binding domain. Expression of *nbzY* in the *ntdY* mutant via pCAR27 restored taxis toward 2NT to approximately wild-type levels after growth in uninduced and 2NT-induced conditions (Fig. 5).

Although the response of the *ntdY* mutant to nitrite was consistently slightly weaker than that of JS42m, suggesting that NtdY might encode a nitrite receptor, the complemented strain (CAR3[pCAR27]) did not show a consistently stronger response to nitrite compared to strain CAR3 (Fig. 5). To test whether overexpression of NbzY might affect direct detection of 2NT, we introduced pCAR27 into the *ntdAc* mutant, which can sense 2NT only directly, as it cannot convert 2NT to nitrite or obtain energy from 2NT. The *ntdAc* mutant expressing *nbzY* via pCAR27 showed very low responses to 2NT after growth under both uninduced and 2NT-induced conditions, similar to those of the *ntdAc*

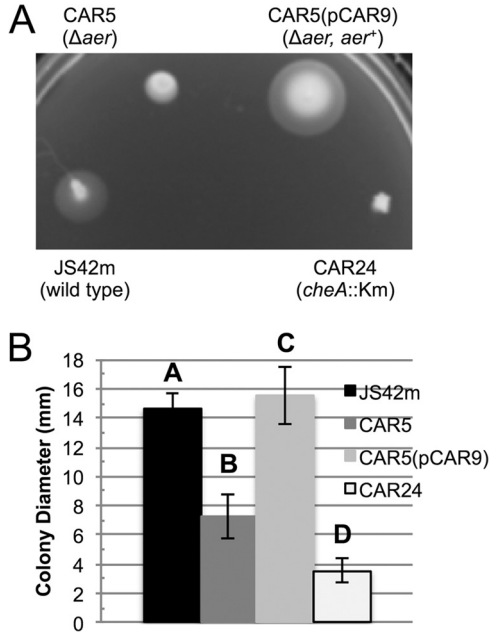

FIG 6 Responses of the wild type (JS42m), *aer* deletion mutant (CAR5), and complemented *aer* mutant (CAR5[pCAR9]) in soft agar plates containing 2 mM 2NT after incubation for 2 days at 30°C. Strain CAR24 (*cheA*::Km) was used as a negative control. (A) Representative 2NT soft agar plate. (B) Averaged colony diameters in 2NT soft agar plates ($n = 6$; error bars represent standard deviations). Different letters indicate significantly different means as calculated by one-way analysis of variance (ANOVA) (Tukey multiple comparison test; $P < 0.05$).

mutant alone (Fig. 5). Therefore, introduction of pCAR27 into this strain did not increase the response to 2NT as might be expected if NtdY/NbzY were a 2NT-specific receptor. While the results do not definitively identify the specific chemoattractant detected, they do demonstrate that NtdY plays a role, either directly or indirectly, in the response to 2NT.

The energy taxis receptor Aer appears to be involved in the response to 2NT. In order to determine whether energy taxis contributes to the metabolism-dependent response to 2NT, we wanted to test whether energy taxis receptors play a role in the response. The product of Ajs_3295 is a homolog of Aer, the energy taxis receptor of *E. coli* (40% amino acid sequence identity); thus, Ajs_3295 was chosen for inactivation. In MSB soft agar plates containing 2 mM 2NT, *aer* deletion strain CAR5 showed a significantly reduced response to 2NT compared to JS42m (Fig. 6). The *aer* mutant also showed a defective response to succinate in soft agar plates but responded normally to adipate, which is also a good carbon source for JS42 (see Fig. S3 in the supplemental material). The response to 2NT was restored in the complemented *aer* mutant (CAR5[pCAR9]) (Fig. 6). In addition, a double-mutant strain lacking both *ntdY* and *aer* (strain CAR12) showed a response to 2NT that was more defective than those of either of the single mutants both in qualitative capillary assays (Fig. 7A) and in soft agar plate assays (Fig. 7B and C). With the exception of the *cheA* mutant, the characteristics of swimming behavior and overall motility of the various mutant strains appeared similar, and doubling times on 2NT were similar for all strains (data not shown), indicating that differences in colony diameters were due solely to taxis defects.

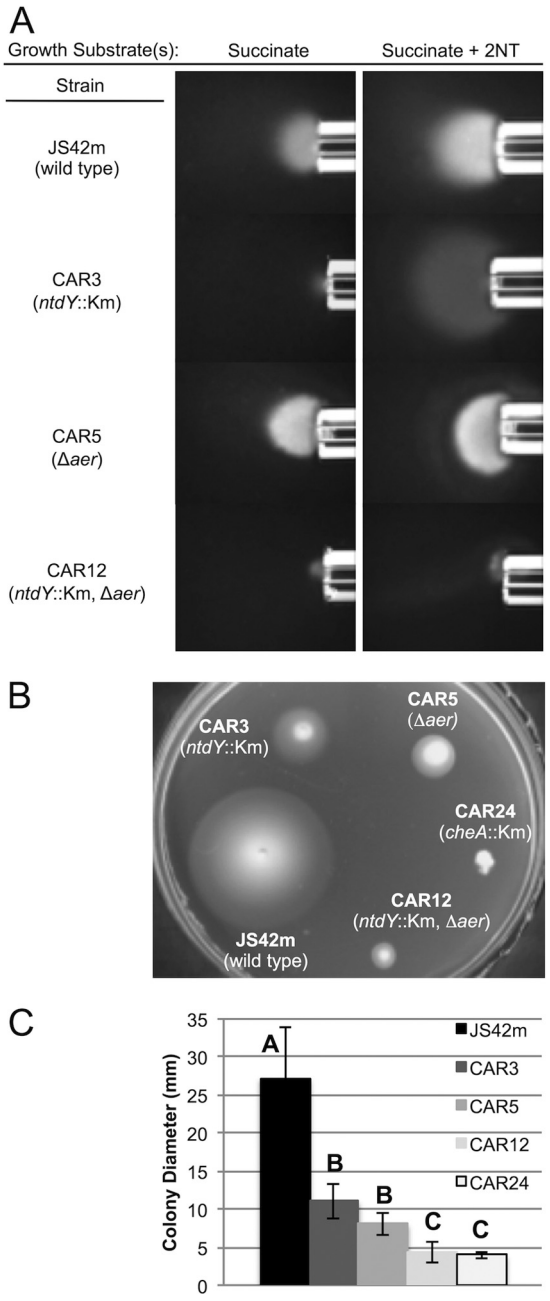

FIG 7 Responses of the wild type and the receptor mutants to 2NT. (A) Qualitative capillary assay results (~4.4 mM 2NT) after growth under uninduced and 2NT-induced conditions. (B) Representative soft agar plate containing 2 mM 2NT after incubation for 3 days at 30°C. Strain CAR24 (*cheA*::Km) was used as a negative control. (C) Averaged colony diameters in 2NT soft agar plates ($n = 4$; error bars represent standard deviations). Different letters indicate significantly different means as calculated by one-way ANOVA (Tukey multiple comparison test; $P < 0.05$).

DISCUSSION

Results presented here provide evidence that the response to 2NT by *Acidovorax* sp. strain JS42m involves constitutive metabolism-independent chemotaxis toward 2NT itself, inducible metabolism-dependent chemotaxis toward the 2NT metabolite nitrite, and inducible metabolism-dependent energy taxis in response to

the cellular energy obtained during growth on 2NT. Previous studies have shown that the *ntd* operon is expressed at a low but significant level in cells containing a functional NtdR even in the absence of inducer (22, 32). Therefore, the constitutive response to 2NT in wild-type JS42m can be attributed to direct detection of 2NT together with the detection of low levels of nitrite and small amounts of energy from 2NT metabolism. The increased response to 2NT by induced cells results from the increased nitrite production and the increased energy generation during fully induced growth on 2NT. Blocking growth at the second step in 2NT degradation resulted in induced and uninduced responses that were lower than those of the wild type but not nearly as low as those of strains blocked at the first step in 2NT degradation, most likely because the *ctdE1* mutant can generate nitrite from 2NT but cannot obtain energy from this substrate. All of our data indicate that the response to 2NT is inducible because expression of the *ntd* operon is inducible. Increased transcription of the *ntd* operon results in the production of more 2NTDO, which increases metabolism of 2NT, generating more attractant (nitrite) and cellular energy.

It was challenging to separate nitrite chemotaxis from the energy taxis that results from 2NT metabolism, because both occur in all induced and uninduced strains that are wild type for 2NT degradation. Separation of these responses was attempted by identification and inactivation of relevant receptors. Deletion of *aer* had a clear effect in 2NT soft agar plates (Fig. 6 and 7). Energy taxis encompasses several types of responses, including pH taxis, redox taxis, light taxis, and aerotaxis. It is a phenomenon in which the cells move toward environments that are more energetically favorable (recently reviewed in references 2 and 52). Energy taxis defects can be observed in soft agar plates (62); in some obligate respiratory bacteria, aerotaxis can mask chemotaxis defects in this assay (3). Unlike many aerobic bacteria that clearly respond to oxygen at the edge of an air bubble (10, 62), we have never been able to observe this response with JS42m. A reduced response in soft agar plates by the *aer* mutant was not seen with all carbon sources (see Fig. S3 in the supplemental material), suggesting that the energy taxis response is substrate specific. Therefore, we interpret the defect in the response by the *aer* mutant to 2NT in swim plates to be a defect in the response to the energy generated from 2NT. A clear case for energy taxis in the response to aromatic growth substrates was demonstrated in *Pseudomonas putida*, as the inactivation of the *aer2* gene resulted in the inability to respond to (methyl)phenols (51). Energy taxis toward nitroaromatic compounds has also been suggested to occur in *Burkholderia* (formerly *Ralstonia*) sp. strain SJ98, which demonstrates a positive tactic response only toward nitroaromatic compounds it can metabolize (9, 39, 49). However, those reports did not address the possibility that a degradation intermediate may be the actual attractant or inducer of the response, and no receptors were identified.

Our results clearly indicate that NtdY plays a role in the response to 2NT. The phenotype of the *ntdY* mutant (Fig. 7A) argues against direct detection of 2NT by NtdY (compare the responses of CAR26 and CAR20 [Fig. 4], strains that are capable only of direct detection of 2NT). Unfortunately, although we favor the hypothesis that NtdY is a nitrite receptor, our results are not definitive. Part of the uncertainty arises from the strong response of the *ntdY* mutant to nitrite when it was provided directly as the attractant, which would indicate that the strain carries at least one additional nitrite receptor. The genome sequence of JS42 encodes 19 MCPs (data not shown), so it is not unreasonable to expect some redundancy in attractant detection such as has been seen previously, for example, in *Pseudomonas aeruginosa* (60, 66).

The phenotype of the double mutant lacking both NtdY and Aer indicates that the two receptors contribute independently to the response to 2NT. The weak response to 2NT by this mutant (Fig. 7A) was similar to that of the *ntdAc* mutant (Fig. 4) and provides further evidence that there is a 2NT-specific chemoreceptor in JS42m that remains to be identified. Relatively few receptors for aromatic attractants have been identified to date. A MCP for detection of naphthalene was identified in *Pseudomonas putida* G7 (14), and one for 2-nitrobenzoate was found in *P. fluorescens* KU-7 (20). Recently, a MCP that confers a strong chemotactic response to toluene was identified in *P. putida* DOT-T1E (28). *Burkholderia* sp. strain DNT and *Burkholderia cepacia* R34, which are capable of growth on 2,4-dinitrotoluene (21, 55), have been reported to be chemotactic toward mono-, di-, and trinitrotoluenes. As yet, no receptors or details about the responses are available, but the responses apparently did not require metabolism (33).

No nitrite-specific receptors that mediate chemotaxis have been identified in bacteria to date. However, another example of chemotaxis to nitrite generated during metabolism has been reported. Cyclic nitramine explosive (CNE)-degrading *Clostridium* sp. strain EDB2 shows taxis toward the CNE compounds RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), and CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) (8). All of these CNE compounds are degraded with the release of nitrite, which is also a strong attractant for strain EDB2. In contrast, structurally related compounds that are not degraded or do not release nitrite did not elicit a response from strain EDB2. Thus, those researchers hypothesized that the strain EDB2 response to CNE compounds is actually a response to the metabolite nitrite (8). However, the report did not confirm that nitrite was generated in detectable amounts during assays and did not identify any relevant receptors.

With the exception of the response to nitrite produced from CNEs by *Clostridium* sp. strain EDB2, taxis toward nitrite has almost exclusively been studied in bacteria capable of using nitrite as a terminal electron acceptor in anaerobic respiration. *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, and *Agrobacterium tumefaciens* all showed taxis toward nitrite only under conditions in which nitrite reductases were active (7, 30). It was concluded that taxis toward nitrite was achieved by detection of nitrite as a terminal electron acceptor, a form of energy taxis (30). An energy taxis receptor containing a CACHE domain (CAlcium and CHEmotaxis [5]) has recently been shown to mediate taxis toward nitrite (among other compounds) in *Shewanella oneidensis* when cells are using nitrite as a terminal electron acceptor (6). *Acidovorax* sp. strain JS42 can use nitrite as a terminal electron acceptor in the absence of oxygen (data not shown), so it is possible that JS42 is capable of responding to nitrite through terminal electron acceptor (energy) taxis. However, all of the taxis assays presented here were carried out under oxic conditions, so we have not addressed this possibility.

The ability of *Acidovorax* sp. strain JS42 to sense and respond to 2NT, which can serve as the sole carbon, nitrogen, and energy source for the strain, is presumably a beneficial characteristic in contaminated environments where concentrations of nitrotolu-

enes may not be uniform. Analyses of the genes, enzymes, and regulation of 2NT degradation in JS42 have suggested that the strain evolved the ability to grow on 2NT recently and subsequently evolved a regulatory mechanism to activate expression of the *ntd* genes in the presence of 2NT (22, 32, 42). Results presented here suggest that JS42 can detect 2NT via two mechanisms that likely preexisted its ability to grow on 2NT: nitrite chemotaxis and energy taxis. Whether the ability to directly sense 2NT resulted from the recent evolution of a 2NT-specific chemoreceptor or is due to fortuitous detection of 2NT by a receptor for a structurally related compound is not yet known; determination of that issue would require the identification and characterization of the 2NT chemoreceptor.

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